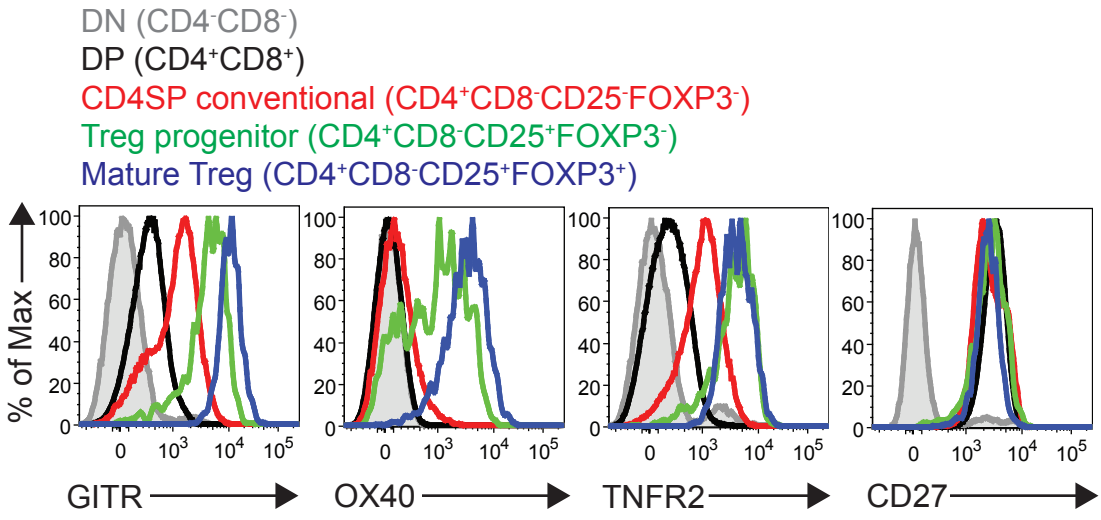
Supplementary Information

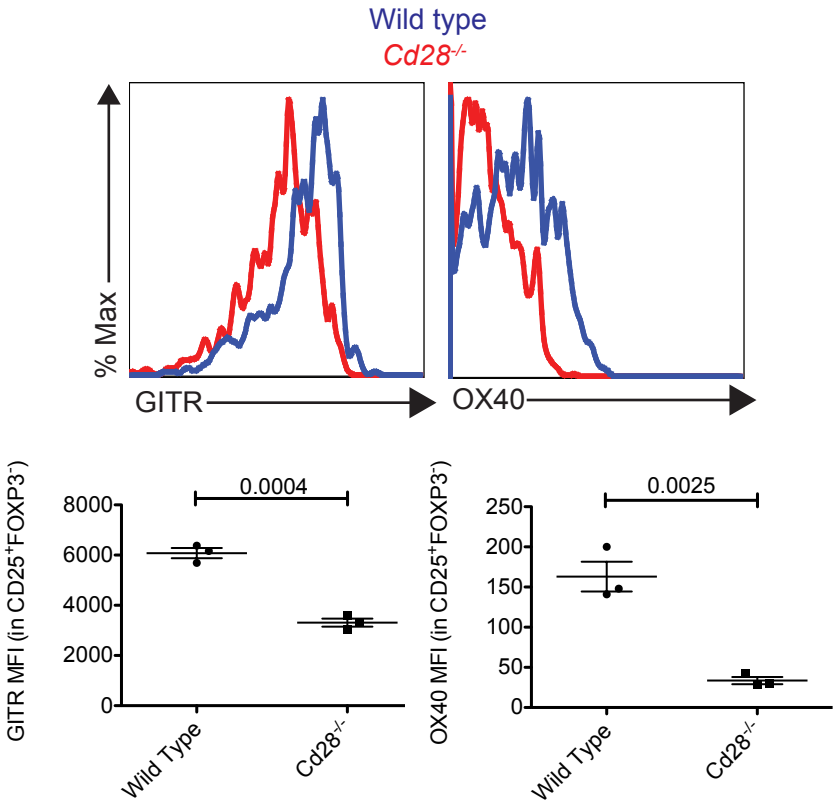
Tumor necrosis factor receptor superfamily costimulation couples T cell receptor signal strength to thymic regulatory T cell differentiation

Shawn A. Mahmud[1], Luke S. Manlove[1], Heather M. Schmitz[1], Yan Xing[1], Yanyan Wang[3], David L. Owen[1], Jason M. Schenkel[2], Jonathan S. Boomer[4], Jonathan M. Green[4,5], Hideo Yagita[6], Hongbo Chi[3], Kristin A. Hogquist[1] & Michael A. Farrar[1]

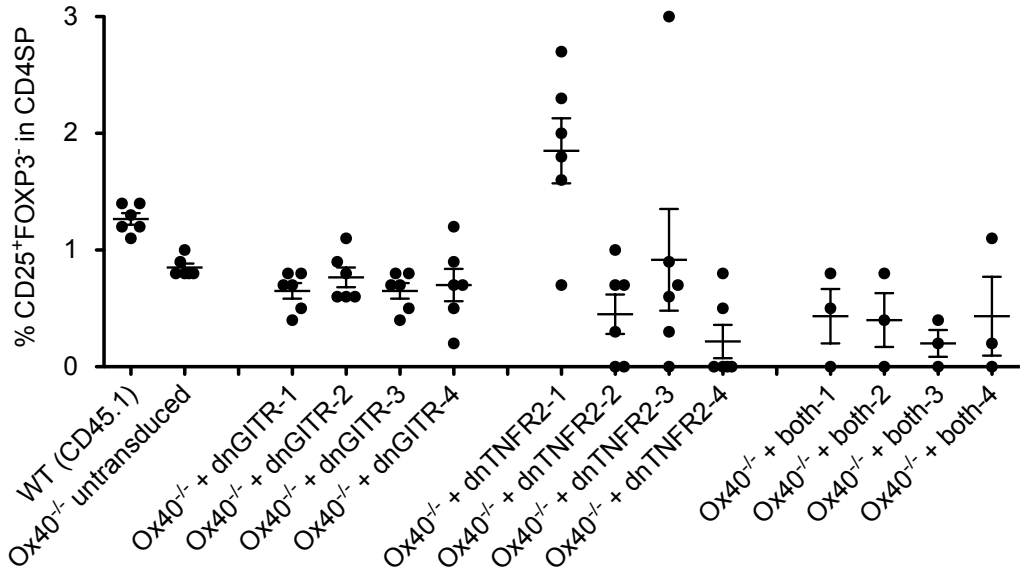


Supplementary Figure S1. TNFRSF expression during thymocyte development.

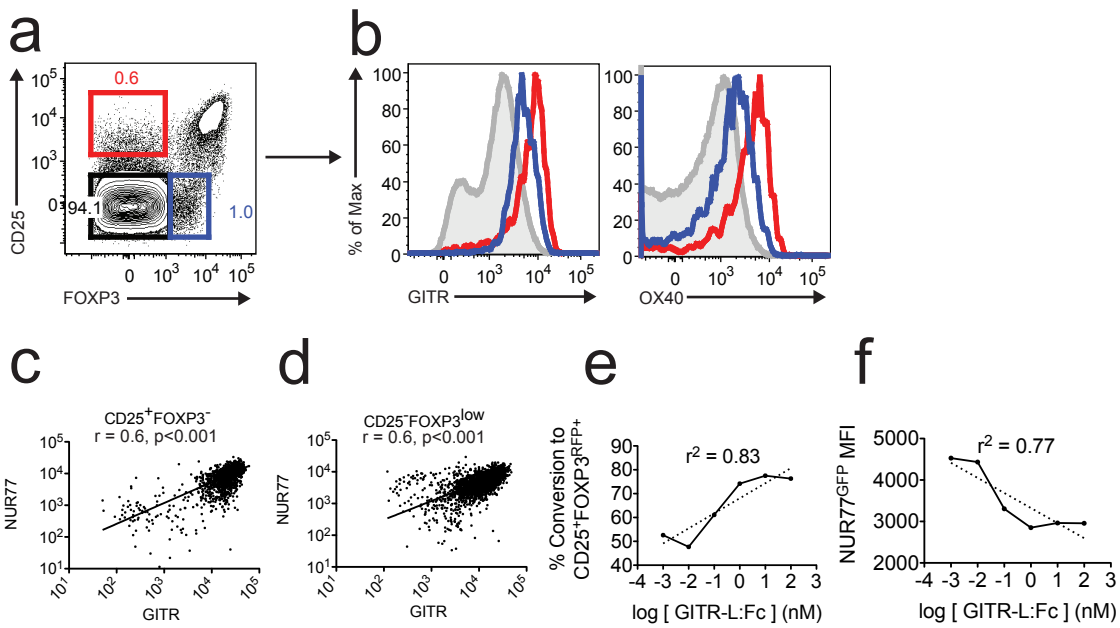
Thymocytes from *Foxp3*^{GFP} reporter mice were harvested and evaluated by flow cytometry for expression of GITR, OX40, TNFR2, and CD27. Gates used to identify the indicated populations were as follows; DN thymocytes: $CD4^-CD8^-$ (grad shaded histograms), DP thymocytes: $CD4^+CD8^+$ (black lines), conventional CD4SP: $CD4^+CD8^-CD25^-FOXP3^-$ (red lines), Treg progenitors: $CD25^+FOXP3^-$ (green lines), and mature Tregs: $CD25^+FOXP3^+$ (blue lines).



Supplementary Figure S2. **GITR and OX40 are reduced on Treg progenitors from *Cd28*$^{-/-}$ mice on the *C57Bl/6* background**. Histograms plotted on the left showing GITR and OX40 expression are derived by gating on Treg progenitors from *Cd28*$^{-/-}$ mice (red histograms) and their wild type littermates (*C57Bl/6* background; blue histograms). In the lower panels, cumulative data are shown for the expression of GITR and OX40 on Treg progenitors from CD28-deficient mice in comparison to wild type *C57Bl/6* littermates (mean ± SEM, n=3, p-values generated by student's T-test).



Supplementary Figure S3. Frequency of $CD25^{+}FOXP3^{-}$ Treg progenitors in dominant negative mixed bone marrow chimeras. Cells in gates drawn in Figure 6b were evaluated for CD25 and FOXP3 expression to determine the frequencies of $CD25^{+}FOXP3^{-}$ Treg progenitors. The percentage of Treg progenitors within CD4SP in each group is plotted as a scatter plot (mean ± SEM, n=6).



Supplementary Figure S4. CD25^{-}FOXP3^{low} Treg progenitors express TNFRSF in proportion to TCR signal strength and are responsive to TNFRSF costimulation.

(**a**,**b**) CD25^{+}FOXP3^{-} Treg progenitors, and the alternately described population of Treg progenitors which are CD25^{-}FOXP3^{low} are gated in red and blue, respectively, and are compared to conventional CD4SP (CD25^{-}FOXP3^{-}; gray shaded histogram) for expression of GITR and OX40. Raw values for GITR and NUR77^{GFP} from (**c**) CD25^{+}FOXP3^{-} and (**d**) CD25^{-}FOXP3^{low} Treg progenitors were plotted and used to calculate Pearson correlation coefficients. P-values assess whether the degree of correlation was statistically significant. (**e**) CD25^{-}FOXP3^{low} Treg progenitors were sorted from *Foxp3*^{RFP} x *Nur77*^{GFP} reporter mice and incubated with 1 U/mL IL2 and increasing concentrations of GITR-L:Fc. The percentage of cells which upregulated CD25 and converted into mature CD25^{+}FOXP3^{+} Tregs after 72h are shown in the scatter plot with a regression line applied. (**f**) The NUR77^{GFP} MFI in newly formed CD25^{+}FOXP3^{+} Tregs is shown after sorting CD25^{-}FOXP3^{low} Treg progenitors from *Foxp3*^{RFP} x *Nur77*^{GFP} reporter mice and stimulating for 72h with 1 U/mL IL2 and increasing GITR-L:Fc.

Table Analyzed	Combined				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	14				
F	23.91				
R squared	0.8428				
ANOVA Table	SS	df	MS		
Treatment (between columns)	250.6	13	19.28		
Residual (within columns)	46.76	58	0.8062		
Total	297.4	71			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
WT (CD45.1) vs $Ox40^{-/-}$ untransduced	1.133	2.186	No	ns	-0.4132 to 2.680
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + dnGITR-1	0.5667	1.093	No	ns	-0.9799 to 2.113
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + dnGITR-4	3.967	7.652	Yes	***	2.420 to 5.513
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + dnTNFR2-1	0.2500	0.4822	No	ns	-1.297 to 1.797
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + dnTNFR2-4	4.350	8.391	Yes	***	2.803 to 5.897
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + both-1	2.833	4.463	Yes	***	0.9392 to 4.727
$Ox40^{-/-}$ untransduced vs $Ox40^{-/-}$ + both-4	4.300	6.773	Yes	***	2.406 to 6.194
$Ox40^{-/-}$ + dnGITR-1 vs $Ox40^{-/-}$ + dnGITR-4	3.400	6.559	Yes	***	1.853 to 4.947
$Ox40^{-/-}$ + dnGITR-1 vs $Ox40^{-/-}$ + both-1	2.267	3.570	Yes	**	0.3725 to 4.161
$Ox40^{-/-}$ + dnTNFR2-1 vs $Ox40^{-/-}$ + dnTNFR2-4	4.100	7.909	Yes	***	2.553 to 5.647
$Ox40^{-/-}$ + dnTNFR2-1 vs $Ox40^{-/-}$ + both-1	2.583	4.069	Yes	**	0.6892 to 4.477
$Ox40^{-/-}$ + both-1 vs $Ox40^{-/-}$ + both-4	1.467	2.001	No	ns	-0.7205 to 3.654

Supplementary Table 1

Statistical analysis of the data sets in Figure 6c-d using ANOVA with Bonferroni comparison.